Recent developments in mucosal delivery of pDNA vaccines

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The development of DNA vaccination to mucosal surfaces has continued apace over the last 2 years, with the investigation of several novel delivery vehicles. There have been advances in the understanding of the basic immunological mechanisms behind the induction of immune responses by plasmid DNA. The mechanistic insights are paving the way for the design of a second generation of mucosally delivered DNA vaccines. This article reviews the recent progress in the field of microparticle, cationic lipid and bacterial delivery systems. All these mechanisms afford some protection from environmental degradation and facilitate DNA uptake. These methods have been compared with respect to transfection efficiency, ability to elicit a full range of immune responses and their relative safety for in vivo applications.

Keywords Cationic lipid, delivery, DNA, microparticle, mucosal, vaccine

Introduction

Mucosal immunization

Infectious diseases are associated with global morbidity and mortality. The majority of these diseases are caused by pathogens that first have to either cross or infect/colonize the mucosa prior to infection of the host. It is therefore logical to assume that pre-established pathogen-specific immunity at the site of entry could help prevent the establishment of clinical disease. Despite this, the Salk polio vaccine is the only commonly administered vaccine that is targeted to specifically generate mucosal, as well as systemic immunity [1].

Optimal induction of specific immune responses at the mucosa have in general been associated with targeting antigens to the specialized sites of the mucosal immune system. Such sites may include the organized lymphoid tissue associated with the gut, rectum, bronchus and nasopharynx or the draining lymph nodes [2]. Antigens presented at these sites induce the priming and homing of IgA-committed B-cells and effector T-cells to a variety of mucosal tissues. Such an approach has the advantage of potentially achieving both systemic immunity and secretion of antigen-specific IgA at distal mucosal sites. In contrast, with few exceptions, systemic vaccination strategies have failed to elicit significant mucosal immunity [3]. However, systemic vaccinations combined with mucosal administration regimes have been reported to boost mucosal immune responses [4,5•]. The large surface of the mucosal tissues is protected primarily by the secretory form of antibodies belonging to the IgA isotype [6]. Secretory IgA (sIgA) has been demonstrated to have neutralizing functions and the potential to block bacterial and viral adherence to epithelia [7]. In addition to sIgA, cellular proliferative and cytotoxic T-lymphocyte (CTL) responses may be essential for virus clearance and prevention of dissemination from the mucosal surface [8,9].

Induction of both cellular and sIgA responses can be achieved by direct delivery of antigens to the mucosal surface. However, mucosal surfaces are highly immunotolerent environments, mainly due to the high antigenic load commonly found at these sites, such as airborne particulates and food-derived peptides [3]. To overcome tolerance, adjuvants, such as cholera toxin sub-unit B (CTB), are often required to elicit potent immune responses to recombinant antigens [10-13]. A number of studies have demonstrated how mucosal immunization with recombinant antigens can induce protection against subsequent viral challenge [14,15].

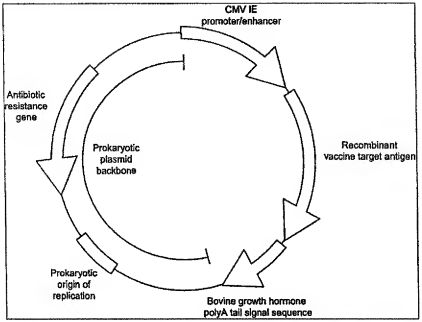
There is currently a need for an alternative to both recombinant protein and attenuated virus vaccines. There have been various risks associated with live attenuated virus vaccines. The secretion of virus has been reported after the administration of live polio vaccine or a combined regime of live and inactivated vaccine [16]. Alternatively, recombinant protein vaccines, often being derived from transformed bacteria, commonly show different protein folding patterns to the native protein. This can lead to the generation of antibodies with only a low affinity to the live pathogen. Furthermore, because of the exogenous nature of the vaccine antigen, immune responses tend to be heavily skewed in favor of T-helper 2 (Th2) responses [3]. The CTL response may also be weaker than following endogenous expression of the antigen from virus infection.

DNA vaccines

A recent development in vaccine technology is that of DNA vaccines, in which antigens are sythesized *in vivo* after direct introduction of their coding sequences into cells. DNA vaccines consist of a bacterial plasmid with a strong eukaryotic viral promoter, the gene of interest and a polyadenylation termination sequence (Figure 1). The immediate early (IE) promoter/enhancer of human cytomegalovirus (CMV) is one of the most frequently used. The IE promoter has been shown to drive high levels of expression of various reporter proteins, in a wide variety of mammalian cell types [17]. DNA vaccines represent a molecularly defined entity, which is both non-replicative *in vivo* (hence safe) and easily produced in large quantities. The safety of DNA vaccines has been studied from the earliest investigations. No *in vivo* evidence for chromosomal integration has been reported using PCR analysis of gel-purified genomic DNA extracted from different tissues [18,19].

Vaccination strategies using plasmid DNA (pDNA)-encoded proteins are particularly pertinent to the generation of immune responses to intracellular pathogens. The DNA-encoded protein

Figure 1. Schematic diagram of a typical DNA vaccine plasmid.



The schematic shows the different sequencing elements commonly found on a typical DNA vaccine plasmid. The cytomegalovirus immediate early (CMV IE) promoter/enhancer sequence initiates RNA polymerase II upstream of the 5' end of the target antigen sequence. The CMV IE sequence will promote transcription in mammalian cells but not prokaryotic. Downstream of the CMV IE sequence is the target antigen sequence orientated 5' to 3', which is flanked by the bovine growth hormone poly adenylation (BGH polyA) signal sequence. The BGH polyA sequence is a highly promiscuous polyA signaling sequence which promotes the synthesis of a 3' polyA tail (important for mRNA stability in mammalian cells). The origin of replication (usually ori E) allows the plasmid to maintain copy number in the prokaryotic transformant (usually *E coli*) used for cloning. Finally, there is a prokaryotic gene which confers antibiotic resistance to the bacterial transformant used for cloning. Many resistance markers can be used, the most common being the *bla* gene which codes for ampicillin resistance in *E coli*. Both the plasmid origin of replication and the antibiotic resistance encoding sequences form part of the prokaryotic plasmid backbone, rich in unmethylated CpG residues.

is expressed in the host cell and therefore undergoes appropriate post-translational modification and intracellular transport. The protein will also be presented by MHC class I and so can generate a CTL response, as well as high affinity humoral responses [20•]. Furthermore, unlike eukaryotic DNA, bacterial DNA contains a high frequency of unmethylated CpG dinucleotide sequences that elicit both innate and adaptive immune responses in vertebrates. These sequences induce dendritic and B-cell activation, upregulation of MHC class II, immunoglobulin and cytokine secretion [21,22,23••]. This means that prokaryotic-derived plasmids containing bacterial DNA backbones, may be natural adjuvants that can increase immune responses to the *in situ* expression of the encoded protein.

Initial studies demonstrated that direct intramuscular (im) injection of pDNA in saline (so called 'naked DNA') using reporter genes such as *luciferase* and *chloramphenicol acetyl transferase* led to long-term expression of the reporter gene [18,24]. Subsequent studies using pDNA encoding viral and bacterial antigens have demonstrated that it is possible to induce immune responses to the encoded protein. [25,26•,27]. Several studies have shown that direct im injection of naked pDNA encoding influenza or HIV proteins protected both mice and non-human primates respectively, after subsequent challenge with homologous or heterologous strains of virus [28,29].

A major drawback with using naked pDNA is that multiple doses of large amounts of DNA (1 to 2 mg) are required to induce optimal CTL and humoral responses in non-human primates [30,31••]. This is further exacerbated by the fact that compared to systemic routes of delivery, such as im inoculation; mucosal administration of naked DNA results in a greatly decreased uptake and longevity [31••]. This is probably due to endonucleases present in and the general dilution effects of mucosal secretions, which may decrease the effective concentration of pDNA at the mucosal surface [32, Klavinskis LS, unpublished observations]. However, recently a number of advances have been made, particularly with respect to pDNA delivery, that have greatly increased the efficacy of mucosal DNA vaccination. The four recent delivery systems that have been applied to mucosal DNA vaccine delivery include: ballistic delivery, cationic lipid complexes, microparticles and bacterial vectors.

Delivery of plasmid DNA to mucosal surfaces

Ballistic/jet delivery

The first significant improvement on manual injection of naked DNA was a technique termed 'gene gun'. The gene gun has been particularly effective at delivering pDNA intradermally. Gold microparticles are coated with pDNA in the presence of spermidine and are delivered to the skin by high velocity bombardment. This results in the

expression of the encoded protein in approximately 10 to 20% of dermal cells at the site of bombardment [33]. The gene gun delivers the pDNA directly into the cytosol and is associated with the induction of both humoral and cellular immune responses at lower concentrations of pDNA than by im injection [34•]. Gene gun inoculation of BALB/c mice with as little as 0.4 μg plasmid encoding influenza hemagglutinin glycoproteins could confer 95% protection against lethal infection with live virus, after just one boost [35]. Dermal gene gun inoculation with vaccine plasmid has been shown to be highly effective at inducing high titer antibody responses to a range of bacterial and viral pathogens [36]. These include inducing long-term protection from lethal rabies challenge after just one boost [26•]. Despite the high efficacy of the gene gun, it is a financially expensive method of vaccination, and to adapt it for induction of mucosal immunity requires highly invasive procedures. Such procedures include direct bombardment of the vaginal mucosa or the surgical exposure of the Peyer's patch prior to bombardment [37,38].

A more practical application of ballistic delivery to mucosal surfaces is the use of a high pressure jet injection (derived from dentistry) for the delivery of pDNA in solution to the buccal mucosa [39••]. The jet technique elicits antigen-specific sIgA in lung lavage and specific serum IgG antibody, predominately of the IgG2a isotype. Potentially this method affords a simple, safe and patient compliant method of mucosal vaccination.

Biodegradable particulate delivery systems

In attempts to overcome enzymatic degradation and improve antigen expression of pDNA, a variety of microparticles have been developed. Unlike gold particles, polymer microparticles, synthesized from poly(lactide-co glycolide) (PLG), are fully biodegradable [40]. These microparticles are produced using a solvent evaporation process. During the synthesis of the microparticles, therapeutic drugs or pDNA present in the aqueous-phase become encapsulated during the formation of the emulsion [41-43]. The PLG microparticles are taken up by cells and release their contents slowly as they are biodegraded (at a rate determined by particle size). The entrapped pDNA is protected from the external environment by the polymer, allowing mucosal administration without fear of degradation. However, the rate of uptake of PLG microparticles across the mucosal epithelium is known to be low (< 0.01%), which may limit the efficiency of this approach.

This technology has been used to engineer an orally administered rotavirus microparticle vaccine for preclinical testing in BALB/c mice [44•]. The DNA expression vector used in this study encoded the VP6 protein encoded by the EW strain of rotavirus under the control of the CMV IE promoter/enhancer. The mice were inoculated orally with PLG microparticles containing either VP6-encoding pDNA or control pDNA. Each mouse received a single dose of particles containing approximately 50 μg of pDNA. Antibodies specific to VP6 were detected in serum from 4 weeks post-inoculation. Antibody titers peaked at 6 weeks post-infection and were still present by 12 weeks post-infection. Low levels of fecal sIgA specific to VP6 were detected only after 6 weeks post-inoculation, which is surprising. Significantly lower virus titers were shed in the vaccinated mice compared to the controls, following virus challenge. However, the duration of virus shed remained the same and it was not possible to evaluate whether the vaccine protected the mice from clinical disease. Important questions still need to be addressed to confirm the efficacy of this new approach to DNA vaccination. However, if protection from clinical disease could be achieved with just a single inoculation, this would represent a major advancement in DNA vaccination.

By modifying the solvent evaporation process, cationic PLG microparticles can be generated [45•]. Either cetyltrimethylammonium bromide (CTAB), dimethyl dioctadecylammonium bromide (DDA) or 1,2-dioleoyl-1,3-trimethylammoniumpropane (DOTAP) were dissolved in the aqueous-phase or the PLG polymer solution prior to particle synthesis. The resultant cationic microparticles could then bind pDNA via ionic interactions between the particle surfaces (which are positively charged) and the negatively charged phosphate backbone of the DNA. From the three types of cationic particle produced, the PLG/CTAB particles had the highest surface charge density and bound pDNA the most efficiently. All three cationic microparticle beads were loaded with pDNA encoding HIV-1 p55 gag, driven off the CMV IE promotor/enhancer. The pDNA-bound microparticles were injected via the im route and compared with an equal dose (1 μg) of naked pDNA encoding HIV-1 p55 [45•].

The naked DNA failed to induce a CTL response after a single inoculation. By comparison, both the PLG/CTAB-p55 pDNA and the PLG/DDA-p55 pDNA induced a potent CTL response, above those observed in p55-expressing vaccinia infected mice. All three types of surface-bound pDNA microparticles induced high serum antibody titer 1 month after the booster vaccination. p55 gag-specific antibody titers were 2 to 3 $\log_{10}$ higher than were induced by naked vaccine vector alone. It has been argued that pDNA has greater stability when bound to the surface of a bead rather than encapsulated. This is due to the high shear that occurs at the organic-/aqueous-phase interface during encapsulation [46]. The *in vitro* release rate of pDNA from the cationic microparticles was initially rapid (35% by day 1) but then slowed until by day 14, 75% of the pDNA bound to the beads had been relinquished. The dynamics of the release properties are potentially closer to an acute virus infection than the encapsulated pDNA delivery systems. However, whether the cationic PLG microparticles offer a more potent mucosal delivery system remains to be seen. Singh *et al* [45•] allude to enhanced mucosal potency in their paper on systemic delivery. The mucosal data is awaited for critical review.

Cationic lipid complexes

Cationic lipid technology has provided one of the best-characterized non-viral delivery systems. The rationale was the ability of these lipids to facilitate transfer of the heavily charged pDNA into the cytosol. The lipids used to complex the pDNA consist of a positively charged lipid, eg, DMRIE [47-50] or GAP-DLRIE [48,51,52], which bind to the negative charge of the phosphate pDNA backbone. A neutral phospholipid is generally included as a colipid for stability. The formation of the pDNA-lipid complexes is in many

respects analogous to the cationic microparticles described previously. Successful gene transfer requires the condensation of the plasmid, cellular-binding and uptake [53]. Many different lipid formulations have been analyzed which enhance these processes in specific cell types [51,54].

Lipid formulations have mostly been applied to the delivery of pDNA vaccines to the respiratory and gastrointestinal tract [31••,49,55,56]. It has now been shown that incorporation of pDNA into lipid complexes can result in enhancement of both humoral and cellular immune responses following mucosal delivery [31,48]. This enhancement of the immune response has in part been attributed to an increase in expression of the encoded protein in mucosal tissues [31••]. Intranasal delivery of a reporter gene (firefly *luciferase*) complexed to DMRIE/DOPE was demonstrated to increase protein expression by 30-fold in nasal tissue compared to administration of the cognate naked pDNA [31••]. Analysis of the immune responses induced to the encoded protein demonstrated that immunization with pDNA-lipid complexes induced a significant increase in specific mucosal IgA antibody when compared with administration of naked pDNA [31••]. Following intranasal delivery of the pDNA-lipid complexes, specific sIgA was detected in vaginal and rectal fluids [49]. This observation provides clear evidence for the potential of mucosal delivery of pDNA at one site to induce immune responses at distal sites via a non-invasive route of vaccination. This may relate to the concept of the common mucosal immune system [57].

As discussed above, the lipids selected for complexing with pDNA may influence the transfection efficiency of specific cell types, and may act synergistically with bacterial pDNA in providing an adjuvant effect [Barnfield C, unpublished observations]. A comparison of humoral responses elicited by either intranasal or oral vaccination with DMRIE/DOPE or Dc Cholesterol/DOPE indicated that both antigen-specific serum IgG and antigen-specific IgA were significantly enhanced using DMRIE/DOPE [Barnfield C, Klavinskis L, unpublished observations]. This observation may relate in part to the level of protein expression achieved and the adjuventicity of the lipids [Barnfield C, Klavinskis L, unpublished observations]. These mechanistic insights into the mode of action of pDNA lipid complexes following mucosal administration, may explain why in certain studies using lipid-formulated pDNA-specific mucosal antibodies were only minimally detected [50,55].

Bacterial vectors

An alternative to lipid complexes or microparticles is the use of plasmid transformed live bacteria to deliver the pDNA to the mucosa. Presently four different bacterial species are being evaluated for pDNA delivery; *Shigella flexneri*, *Salmonella typhi*, *Listeria monocytogenes* and invasive strains of *Escherichia coli* [58]. Integration of the pDNA into the genomes of cell lines following *in vitro* infection with pDNA transformed *E coli* and *L monocytogenes* have been reported. However, it is not known whether integration occurs *in vivo*. Clearly safety concerns will have to be fully addressed prior to clinical trials using bacterial pDNA delivery vectors in man.

A highly attenuated strain of *Shigella flexneri* has been developed and applied to deliver a measles virus (MV) DNA vaccine in a murine study [59••]. The *Shigella flexneri* strain used contained a deletion (Δ) in the *asd* gene. This gene encodes an aspartate β-semialdehyde dehydrogenase, which is essential for cell wall synthesis and bacterial growth. Between 10^7 and 10^8 colony forming units (cfu) could be safely inoculated (in) into the lungs of both normal and immunocompromised (γ-interferon knockout and severely combined immunodeficient) BALB/c mice. Despite being replication defective, the *Δasd* mutants retain the ability to escape the lysozome and deliver the pDNA directly into the cytosol, following cellular uptake [60]. The bacteria were cleared from the lungs of all the mice within 3 days of inoculation, demonstrating the lack of reversion back to virulence by the *Δasd* mutants. Conversely, similar titers of wild-type *Shigella flexneri* inoculated into the same three strains of mice proved 100% lethal by 5 to 6 days post-inoculation.

For the purposes of vaccination the *Δasd Shigella flexneri* mutants were transformed with a DNA vaccine plasmid encoding either the *MV fusion, hemagglutinin* or *nucleoprotein* gene driven by the CMV IE promotor/enhancer. Test mice were inoculated intranasally, with 1 to 3 x 10^7 cfu and then boosted monthly. The immune responses induced to the MV-encoded proteins were predominantly but not exclusively Th1, since IL-4 was produced by splenocytes following *in vitro* restimulation with the appropriate MV protein. Both IgG and IgA responses to MV were detected in the serum of vaccinated mice, with titers increasing after the second boost. Antibody titers however, remained relatively low. Conversely, vaccination generated strong CTL responses after one boost. Surprisingly, the CTL responses were equivalent to the levels seen in mice inoculated by the systemic route with attenuated *Salmonella typhi* harboring the same MV-encoding plasmid. Furthermore, immune responses were only slightly reduced in mice vaccinated against *Δasd Shigella flexneri* prior to vaccination with MV strains. This study clearly highlights the potential of using bacterial DNA vaccine delivery systems, especially with respect to the third world.

Conclusion

The possibility of introducing mucosal immune responses to a protein expressed directly from an introduced gene by various modes of pDNA delivery represents an attractive alternative to attenuated or recombinant vaccines. Mucosal delivery of pDNA vaccines by intranasal, buccal or oral routes represent simple, non-invasive routes which are highly suited for mass vaccination. The immune responses induced after mucosal delivery of DNA vaccines have in general been lower than those induced after parental delivery. However, with improved mechanisms of delivery, increased antibody as well as cellular responses can now effectively be induced. A number of new delivery techniques are being employed which enhance antigen expression and subsequent immune responses, ie, microparticles, cationic lipid aggregates and bacterial carrier vectors. It is becoming apparent that the type of immune response elicited by mucosal application of pDNA is influenced by several factors including the mucosal site, delivery vehicle and incorporation of adjuvants. The second generation of DNA vaccines both protect pDNA from mucosal endonuclease degradation and enhance cellular uptake. With the exception of the Salk polio vaccine [1],

there is currently an absence of clinically administered mucosal vaccines. The increased efficacy of mucosal DNA vaccines may make these vaccines realistic candidates for clinical trials in the future.

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